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Deficiency of Two Red-Cell Flavin Enzymes in a Population in Sardinia: Was Glutathione Reductase Deficiency Specifically Selected for by Malaria?

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Summary

In two areas in Italy where malaria was endemic—in the Po delta and Maremma on the west coast—we have found a high prevalence of an inherited flavin-deficient red cell in the normal population, suggesting selection by malaria. This study in Sardinia enabled a direct comparison of red-cell activities of FAD-dependent glutathione reductase (EGR) and FMN-dependent pyridoxine phosphate (PNP) oxidase in an ethnically homogeneous population, between two coastal villages where malaria was endemic from 300 B.C. and two mountain villages with no history of malaria. Both enzyme activities were significantly lower on the coast, and it did not seem that this could be explained by possible small differences in dietary riboflavin. As was thought to be the case in Ferrara and Grosseto, it is probable that a genetically controlled flavin-deficient red cell was selected for by malaria. Low EGR apoenzyme activity was more common on the coast, usually explaining the accompanying low basic EGR activity, and may also have been selected for by malaria. This adds to evidence from others that the mechanism of defence of a flavin-deficient red cell against malaria may be through EGR deficiency. It could also play a part in the protection given by heterozygous β-thalassemia. The multifactorial protection of the population against malaria is discussed.

Introduction

There is good evidence that flavin-deficient red blood cells give protection against severe malaria. Inhibition of multiplication of the malaria parasite in red cells has been demonstrated both in vivo and in vitro in animals and humans in the presence of nutritional or experimental flavin deficiency (Seeler and Ott 1944; Rama Rao and Sirsi 1956; Kaikai and Thurnham 1983; Oppenheimer et al. 1983; Thurnham et al. 1983; Dutta et al. 1985, 1986, 1988; Bates et al. 1986; Das et al. 1988, 1990; Dutta 1991). It is still uncertain what the mechanism of defence is, but a very significant reduction in parasite multiplication has been demonstrated in vitro in red cells in which the flavin enzyme, FAD-dependent glutathione reductase (EGR), has been inhibited by nitrosoureas (Zhang et al. 1988*a*, 1988*b*). The reduction in multiplication of parasites seemed to be due to poor reduced glutathione (GSH) regeneration, which would lessen protection against increased oxidative stress in parasitized red cells.

We have found a high incidence of flavin-deficient red cells, not due to dietary deficiency, in two areas in Italy in which malaria was endemic in the past—in Ferrara province in the Po delta (Anderson et al. 1993), where malaria was endemic from the 12th century, and in the Grosseto area of Maremma, of Etruscan fame, on the west coast (Anderson et al. 1994), where malaria was endemic from 300 B.C. Red-cell flavin deficiency was demonstrated indirectly by low activities of FMN-dependent pyridoxine phosphate (PNP) oxidase and/or FAD-dependent EGR activity. There is strong evidence of a genetic control, which was shown in many family studies in Ferrara in which PNP oxidase activities were evaluated (Anderson et al. 1989, 1993) (the more sensitive measure of a flavin-deficient red cell [Rasmussen et al. 1980; Anderson et al.1989]), but, since the families are incomplete, there is insufficient data for statistical analysis (Anderson et al. 1994). However, the findings are very suggestive of a genetic selection by malaria. Malaria was also endemic from 300 B.C., at the latest, in many parts of Sardinia, particularly on the coast but not in the mountains, and therefore it was possible to test a selection theory by direct comparison. A model for this was provided by the classical study by Siniscalco et al. (1961), in which they found that the prevalence of the genetic blood diseases, β-thalassemia and glucose-

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Table 1

Distribution of α- and β-Thalassemia, G6PD Deficiency, and Normal Phenotype in Two Mountain and Two Coastal Villages in Sardinia

	No. of Individuals in					
	Mountain Villages			Coastal Villages		
	Desulo	Tonara	Total	Orosei	Galtellì	Total
Heterozygous α-thalassemia	4	2	6 (13%)	7	11	18 (33%)
Heterozygous β-thalassemia	1	2	3 (6%)	5	4	9 (17%)
G6PD deficiency	0	2	2 (4%)	2	1	3 (6%)
Normal	20	16	36 (77%)	12	12	24 (44%)
Total	25	22	47 (100%)	26	28	54 (100%)

6-phosphate dehydrogenase (G6PD) deficiency, was significantly higher in two coastal villages than in two mountain villages, suggesting selection by malaria.

We planned a pilot study in these same villages, to measure red-cell flavin status in 101 school children not yet screened for thalassemia and G6PD deficiency. Since it is possible that the red-cell flavin status could be different in the mountains than on the coast if their diets were widely different, a questionnaire about their diets, with special reference to riboflavin content, was compiled to investigate this. PNP oxidase and EGR activities in normal children and in children with heterozygous α- and β-thalassemia are reported in this paper.

Subjects, Material, and Methods

Subjects

The project involved 101 school children 11–16 years of age who had not previously been screened for thalassemia or G6PD deficiency. The children came from four villages in central Sardinia, and, as far as could be known, their origins were in these villages. Two of the villages, Tonara and Desulo, were in the mountains, where there was no history of endemic malaria, and two other villages, Galtellì and Orosei, were on the east coast, where malaria was endemic from 300 B.C., at the latest. It had been well established in these four villages, used in the study by Siniscalco et al. (1961), that the populations were ethnically homogeneous. The high numbers of children, in particular on the coast, with the previously undiagnosed genetic blood diseases (table 1), as well as the surprising number with α-thalassemia, meant that there were only 36 of 47 from the mountains and 24 of 54 from the coast in whom the red-cell flavin enzymes could be compared between mountains and coast in normal children.

Blood Collection

The project was organized by Dr. Laura Corda of Centro per le Microcitemie, Nuoro, who had personal contact with the teachers in each school. Blood samples were collected at the schools by the first four authors of the present paper and were brought back to Nuoro, where the diagnosis of α- and β-thalassemia and G6PD deficiency was done in Dr. Corda's laboratory. Hemolysates for enzyme assays were made on heparinized blood, and, after storage at −80°C, PNP oxidase and EGR activities were assayed in London and Ferrara, respectively.

Material

NADPH, FAD, and glutathione, oxidized form, were obtained from Sigma Chemical. All other reagents were obtained from British Drug House.

Methods

EGR activity.—This was measured in vitro with and without addition of FAD, in order to assess red-cell flavin status, using the method of Bayoumi and Rosalki (1976) with small modifications as described elsewhere (Clements and Anderson 1980*a*; Anderson et al. 1987*a*). The lower limit of normal of the basic activity measured without added FAD was 160 μmol NADPH/10^{12} red cells/min.

PNP oxidase activity.—This was measured as described elsewhere (Clements and Anderson 1980*b*), and the lower limit of normal was 10 nmol pyridoxal phosphate/g hemoglobin (Hb)/h. The setting of the lower limits of normal of the two enzyme activities has been described in detail elsewhere (Anderson et al. 1994).

Dietary questionnaires.—Since there were no facilities for measuring dietary riboflavin intake (DRI) in the children, a questionnaire was designed by one of us (M.G.), to detect any differences in dietary riboflavin between the mountain and coastal villages. The frequency with which foods were habitually eaten was graded in the following categories, without reference to the amount: (0) never, (1) once a year, (2) once a month, (3) at least once a week, (4) more than once a week, (5) at least

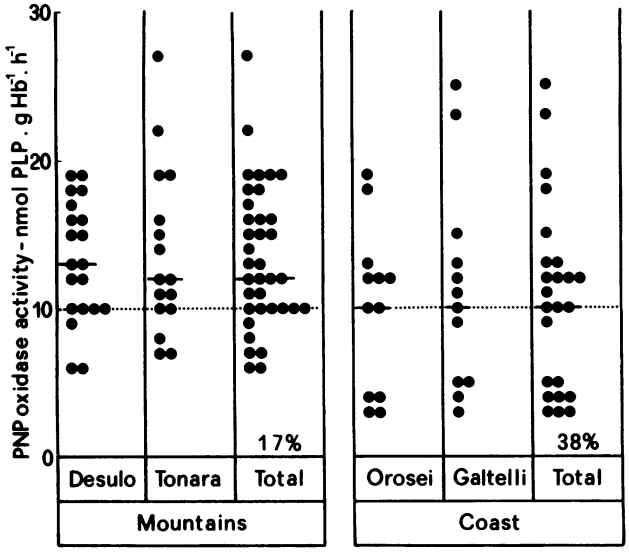

Figure 1 Red-cell PNP oxidase activity in well-nourished normal Sardinian school children from two mountain villages—Desulo (20 subjects) and Tonara (16 subjects)— where there had been no malaria and from two coastal villages—Orosei (12 subjects) and Galtellì (12 subjects)—where malaria was endemic from 300 B.C.. The horizontal bars represent the median red-cell PNP oxidase activity, and the dotted lines represent the lower limit of normal PNP oxidase activity. The percentages represent the prevalence of low PNP oxidase activity. Overall, activities were significantly lower on the coast ($P < .05$) (mean ± SD: mountains 13.5 ± 4.7; coast 10.5 ± 6.2).

once a day, and (6) more than once a day. The answers from each child in the four villages were analyzed.

Screening for α- and β-Thalassemia and G6PD Deficiency

This screening included a blood count and film, and measurement by standard methods, of red-cell fragility, Hb electrophoresis, percentage of Hb A2 and Hb F, serum iron, and red-cell Hb H inclusion bodies. The criteria for diagnosis were those established by Weatherall and Clegg (1980). The screening for G6PD deficiency was done by the method of Beutler and Mitchell (1968).

Statistical Methods

The Wilcoxon nonparametric test for nonpaired data was used for determining the significance of the difference between two groups; and linear regression and correlation analysis were used for determining the significance of the relationship between two variables.

Results

Red-Cell PNP Oxidase and EGR Activities in Normal Children in Mountain and Coastal Villages

There was no significant difference between the mountain villages or between the coastal villages in either enzyme activity (figs. 1 and 2). In combined findings for the mountains and for the coast, both enzyme activities were significantly lower on the coast ($P < .05$ and $P < .01$, for PNP oxidase and EGR, respectively) and were similar to those found in the other malarial areas, Ferrara and Grosseto (Anderson et al. 1993, 1994). The prevalence of low PNP oxidase and EGR activities was considerably higher on the coast (38% and 29%, respectively) than in the mountains (17% and 19%, respectively). In particular, there were eight subjects from the coast who had a considerably lower PNP oxidase activity, and five who had lower EGR activity, than was found in the mountains. In addition, the percentage of EGR stimulation by FAD was significantly higher on the coast ($P < .05$) (results not shown), confirming the greater red-cell flavin deficiency.

There was, as reported elsewhere (Clements and Anderson 1980*a*; Anderson et al. 1987*b*), a significant correlation overall between the activities of the FMN-dependent PNP oxidase and FAD-dependent EGR (mountains, $P < .05$; coast, $P < .02$). However, as was also found in Grosseto, some subjects had a low EGR apoen-

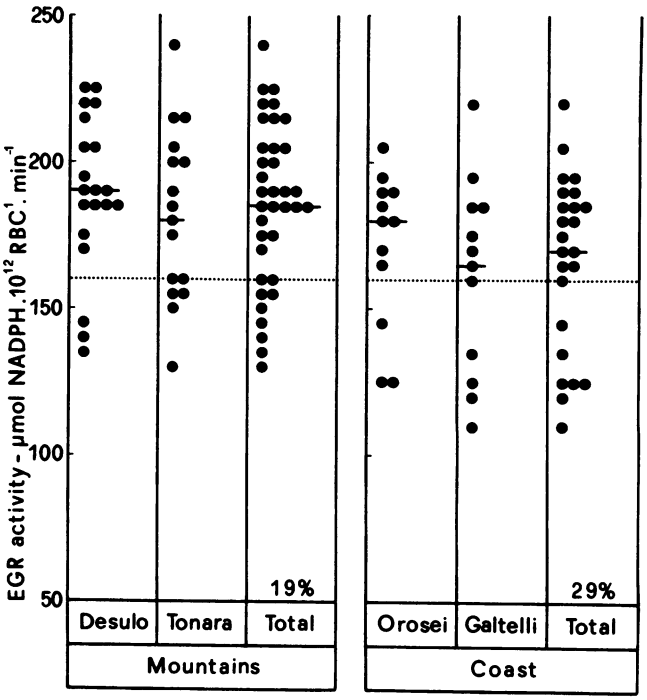

Figure 2 Basic EGR activity in well-nourished normal Sardinian school children from two mountain villages—Desulo (20 subjects) and Tonara (16 subjects)—where there had been no malaria and from two coastal villages—Orosei (12 subjects) and Galtellì (12 subjects)—where malaria was endemic from 300 B.C.. The horizontal bars represent the median EGR activity, and the dotted lines represent the lower limit of normal EGR activity. The percentages represent the prevalence of low EGR activity. Overall, activities were significantly lower on the coast ($P < .01$) (mean ± SD: mountains 186.2 ± 27.4; coast 166.0 ± 30.1).

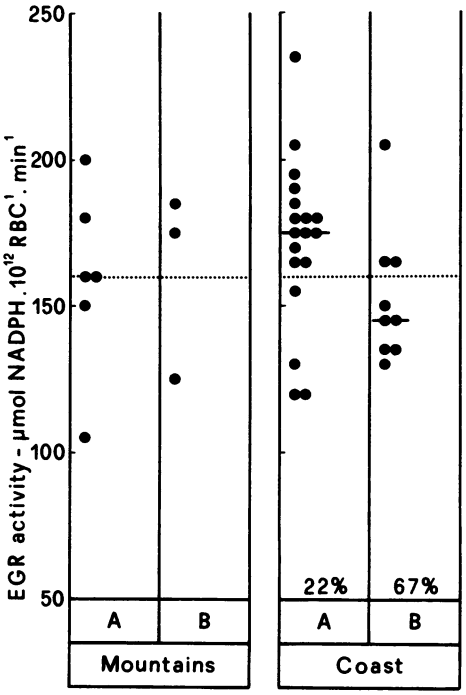

Figure 3 Basic EGR activity in well-nourished Sardinian schoolchildren who either were from two mountain villages where there had been no malaria and who had (*A*) heterozygous α-thalassemia (6 subjects) and (*B*) heterozygous β-thalassemia (3 subjects) or who were from two coastal villages where malaria was endemic from 300 B.C. and who had (*A*) heterozygous α-thalassemia (18 subjects) and (*B*) heterozygous β-thalassemia (9 subjects). The horizontal bars represent the median EGR activity, and the dotted lines represent the lower limit of normal EGR activity. The percentages represent the prevalence of low EGR activity. On the coast, where numbers permitted statistical analysis, activities were significantly lower in β-thalassemia ($P < .01$) (mean ± SD: *A*, 172.0 ± 28.7; *B*, 152.3 ± 22.5).

zyme (≤200 µmol NADPH/10^{12} red cells/min). In the majority of these subjects (seven total; two (5.6%) from the mountains and five (20%), one of whom had heterozygous α-thalassemia, from the coast), basic EGR activity was very low, whereas PNP oxidase was low in only one, was borderline in one, and was normal in five of them, so that the majority (of the seven) fell outside the correlation. In most of these subjects, therefore, the low basic EGR activity was probably mainly due to the deficiency of EGR apoenzyme activity, rather than to a deficiency of FAD. EGR apoenzyme activity overall was significantly lower on the coast ($P < .05$).

EGR Activities in Heterozygous α- and β-Thalassemia

In the coastal area, where numbers allowed statistical comparison, there was no significant difference in basic EGR activity between α-thalassemia heterozygotes and the normal subjects (fig. 3), whereas in subjects with β-thalassemia EGR activity was significantly lower ($P < .05$) than in that in the normal subjects, as had been found previously in Ferrara (Anderson et al. 1987*b*, 1989, 1993).

Dietary Questionnaires

The dietary questionnaires showed that the majority of children ate well, including foods that contain riboflavin. Almost all children from the mountains and almost all children from the coast ate fruit and bread (which contain significant but low amounts of riboflavin) more than once a day and ate cheese and meat (which are high in riboflavin, particularly the cheeses) regularly, i.e., from more than once a week to daily. Pasta and vegetables were eaten only a little less regularly, by all.

However, there were differences in consumption of some foods. In general, it appeared that in the mountains considerably more milk was drunk and that more of some of the foods containing riboflavin—i.e., fish (weekly), eggs (weekly), vegetables, legumes, and minestrone—were eaten and that marginally more yogurt was eaten. On the other hand, in the coastal areas more ham, chocolate milk drinks, meat extracts, pizzas, and marginally more offal, all foods also containing riboflavin, were eaten.

Milk is probably the primary source of riboflavin if it is drunk in quantities (see table 2). Thirty-five percent of the children from the coast never drank milk, suggesting a low lactase activity which is known to discourage milk drinking, whereas only 17% of children in the mountains were non–milk drinkers. However, the mountain villages differed from each other—in Desulo all drank milk regularly, but in Tonara 6 (38%) of 16 never drank milk, a proportion that was no different from that for the coast as a whole. However, twice as many in the mountains as on the coast (74% vs. 39%) drank milk at least once a day. On the other hand, most of the latter group regularly ate at least cheeses and meat and often seemed to compensate with ice cream and chocolate drinks.

Although DRI was not measured and therefore a statistical analysis cannot be done, it is interesting to look at the diets of the 11 children from the coast, referred to earlier (figs. 1 and 2), who had lower activities of one or both flavin-dependent enzymes than were found in children from the mountains. Table 2 shows the frequency with which the most important riboflavin-containing foods were commonly eaten. All children ate meat of some sort at least once a day, and all but one ate cheese (seven of them ate cheese daily). Four children (subjects 6, 7, 10, and 11) did not drink milk, but all these ate meat daily, and all but one ate cheese, although only one ate cheese daily. In only one child (subject 6) does it seem that the diet could be low in riboflavin. Four

Table 2

Riboflavin-Containing Foods Eaten by 11 Children from the Coastal Villages, in Whom PNP Oxidase and/or EGR Activities Were the Lowest Found in the Study (Figs. 1 and 2)

Subject	PNP Oxidase Activity[a] (nmol PLP/g Hb/h)	EGR Stimulation[b] (%)	EGR Activity (μmol NADPH/10^{12} red cells/min)		Riboflavin (mg/100 ml)[e]–Containing Foods Eaten[f]											
			Basic[c]	Apoenzyme[d]	Milk (.18)	Yogurt (.17)	Cheese[g] (.11–.83)	Meat (fresh, .04–.35; preserved, .18–.26)	Offal[h] (.18–3.30)	Fish (.04–.26)	Eggs (.31)	Pasta (.11–.19)	Vegetables[i] (.02–.58)	Ice Cream (.1)	Chocolate Drinks (.07–.39)	Meat Extracts (.1)
1	2.6	95	123	239	4	0	5	5	2	3	2	5	4	4	4	4
2	2.9	59	172	273	6	2	5	6	1	3	3	5	4	1	4	3
3	3.2	21	123	149	3	3	5	5	0	4	3	3	4	6	6	...
4	3.5	44	191	276	6	4	5	5	1	2	3	4	4	3	4	2
5	3.6	41	190	268	3	4	5	5	2	3	3	4	6	3	4	4
6	4.0	77	163	288	0	0	0	5	0	0	3	4	4	3	0	0
7	4.8	76	135	240	0	0	4	6	1	3	2	4	...	1	3	3
8	5.0	71	158	271	4	0	3	6	3	4	2	4	5	1	4	4
9	8.9	60	109	175	5	3	5	5	2	3	3	5	4	2	1	4
10	10.3	70	119	202	0	0	3	6	0	2	0	4	0	1	4	3
11	12.2	50	124	186	0	0	5	6	3	0	2	5	5	1	3	5

[a] Deficiency: <10.
[b] Deficiency: >40.
[c] Deficiency: <160.
[d] Deficiency: <200.
[e] National Institute of Nutrition (1987) tables of food composition.
[f] Nos. indicate frequency with which a particular food is eaten: 0 = never; 1 = at least once a year; 2 = at least once a month; 3 = at least once a week; 4 = more than once a week; 5 = at least once a day; and 6 = more than once a day.
[g] Cheeses commonly eaten tended to have the higher riboflavin values: mean, 0.36 mg/100 ml.
[h] Liver, 3.3 mg/100 ml; kidney, 2.25 mg/100 ml.
[i] In order of magnitude, with riboflavin values >0.2 mg/100 ml: green "radicchio," spinach, mushrooms, endive, asparagus, broad beans, chilies, broccoli, and parsley.

children (subjects 3 and 9–11) had low EGR apoenzyme activity, which was probably the main reason for a low basic EGR activity; and two of these four did not drink milk. The most important point is that the five children (subjects 1–5) with the lowest PNP oxidase activities in the study all drank milk to a greater or lesser degree and ate cheese and meat daily, and most of them had ice cream and/or chocolate drinks and/or meat extracts fairly frequently, particularly subjects 1 and 3, who had very low activities of both enzymes.

This seems to demonstrate that, in general, the children in the coastal villages with the low activities did not have riboflavin-poor diets. Furthermore, it does not appear that the children with high enzyme activities, seen more frequently in the mountains, had diets richer in riboflavin. Therefore, despite a lower incidence of milk drinking, the lower enzyme activities on the coast cannot be attributed to a riboflavin-deficient diet.

Discussion

It was an advantage in Sardinia that a controlled study in an ethnically homogeneous population could be done on the effect of a long history of exposure to malaria. Thus, the activities of the two red-cell flavin enzymes, FMN-dependent PNP oxidase and FAD-dependent glutathione reductase, in normal children are significantly lower on the coast, where malaria was endemic for centuries (figs. 1 and 2), than in the mountains, where there was little or no malaria. However, it had to be established that the diets in the coastal and mountain areas were not widely different in riboflavin content. In fact, it appeared that the diets might be a little richer in riboflavin in the mountains, particularly as regards milk drinking. However, we found in Grosseto where DRI could be measured, that lack of milk drinking did not usually lead to a low riboflavin intake (B. B. Anderson, unpublished observations).

In general, children on the coast in Sardinia had good diets, for, although less milk was drunk than in the mountains, most of them ate the other riboflavin-containing foods—meat, cheese, and other foods. Thus, it is fairly certain that the lower flavin-enzyme activities found on the coast are genetically determined and not the result of dietary deficiency of riboflavin (table 2); and there is very good reason to believe that the high prevalence of a flavin-deficient red cell found on the coast in Sardinia, where malaria was endemic for many centuries, was due to selection by malaria, as is thought to be the case for Ferrara and Grosseto (Anderson et al. 1993, 1994).

However, from this Sardinian study it is becoming clear that, although there is a significant correlation between EGR and PNP oxidase activities, suggesting a deficiency of flavokinase (the enzyme that converts riboflavin to FMN), there are also other factors affecting their activities. The most important is a low EGR apoenzyme activity (also found in Grosseto), which is mainly responsible for an accompanying low basic activity, and it seems that, since the prevalence (20%) is considerably higher on the coast, a low EGR apoenzyme activity might also have been selected for by malaria. Second, in some of the subjects with a low PNP oxidase activity, basic EGR activity is normal, as seen in three children in table 2. This can be explained, because it is known that, in the situation of flavin deficiency, FAD may be conserved at the expense of FMN (Fass and Rivlin 1969; Prentice and Bates 1981*a*, 1981*b*; Anderson et al. 1987*b*), provided that there is no defect in FAD-pyrophosphorylase, the second enzyme in the riboflavin metabolic pathway, which converts FMN to FAD. Third, theoretically there could be a defect in this second enzyme, which would lead to a high FMN and low FAD and, hence, to normal or high PNP oxidase activity and low basic EGR activity, which can be found for a different reason in the situation of a low EGR apoenzyme activity, already discussed. The only evidence of the former was found in a few subjects with a high PNP oxidase activity inconsistent with a borderline basic EGR activity.

The mechanism by which red-cell flavin deficiency protects against malaria remains uncertain. However, the preliminary finding of a higher incidence of a low EGR apoenzyme activity in the coastal villages adds to previous evidence (Zhang et al. 1988*a*, 1988*b*; Das et al. 1990) that it might occur through low functional EGR activity. This could result from a dietary riboflavin deficiency or from genetic deficiencies of either the flavin EGR coenzyme or the EGR apoenzyme; a resulting impaired GSH regeneration could lessen protection for the parasite against increased oxidative stress. It is possible that this also plays a part in the protection against malaria given by heterozygous β-thalassemia, in which condition the prevalence and severity of EGR deficiency are even higher than they are in the normal population in a malarial area (fig. 3; Anderson et al. 1987*b*, 1989, 1993). This latter is probably because the coenzyme, FAD, in addition to having a genetic deficiency, is diverted to the methemoglobin reductases, which are commonly much increased in heterozygous β-thalassemia (Perry and Anderson 1991). It is interesting that, in contrast, in α-thalassemia, which was very common (33% prevalence) on the coast, the degree of deficiency of EGR was similar to that in normal subjects. Finally, it is central to severe G6PD deficiency that there is a deficiency of functional EGR activity due to lack of NADPH (Kirkman et al. 1975) which leads to low GSH regeneration (Anderson et al. 1987). Therefore, the protective mecha-

nism against malaria may be the same in G6PD deficiency and in the flavin-deficient red cell.

In Sardinia, on the coast, where malaria was endemic, 42 (78%) of the 54 children studied had one or more of the protecting factors discussed above. We have put forward a hypothesis that a protecting factor throughout the world in times of antiquity was primary adult lactase deficiency (Anderson and Vullo 1994). The fact that less milk is drunk on the coast than in the mountains suggests a higher prevalence of lactase deficiency where malaria was endemic, and therefore lactase deficiency may also have been selected for by malaria in Sardinia. This needs to be confirmed by direct measurement of lactase activity.

The probability of being infected by malaria is very high for an individual living in an area where malaria is endemic, and the disease can be very severe. Therefore, in order to survive in a malarial area in the past, it would have been necessary for an individual to have one or more genetic factors protecting against severe malaria. This might be why in Sardinia the population has survived in an ethnically pure form (e.g., there is only one β-thalassemia mutation), whereas the many invaders over the centuries could have been wiped out by malaria.

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